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HIF-1/VP16 Gene Therapy Enhances the In-Vitro and In-Vivo Angiogenic/Arteriogenic Effects of Marrow-Derived Stromal Cells

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Abstract

Background Marrow-derived stromal cells (MSCs) release many arteriogenic cytokines and augment the collateral response to ischemia when delivered locally. Furthermore, cell transduction with the hypoxia inducible factor-1α/VP16 construct (HIF-1α/VP16) increases expression of hypoxia-dependant genes. Thus, constitutive overexpression of HIF-1α/VP16 may further enhance the arteriogenic effects of MSCs. **Methods and Results** MSCs from patients undergoing stem-cell therapy were either transduced with the Ad.HIF-1α/VP16 vector, or exposed to normoxia or 1% O_2. VEGF and bFGF levels increased significantly in the conditioned media (MSC^{CM}) from HIF-1α/VP16-transduced MSCs compared to controls. When compared to MSC^{CM} from MSCs under normoxia or hypoxia, MSC^{CM} from transduced MSCs increased endothelial and smooth muscle cell proliferation and migration. In a murine model of hindlimb ischemia, adductor muscle injection of HIF-1α/VP16-transduced MSCs increased collateral perfusion, improved limb function and reduced calf muscle atrophy compared to non-transduced cells. Adductor muscle levels of VEGF were significantly higher in those mice receiving HIF-1α/VP16-transduced MSCs compared to controls. **Conclusions** Constitutive over-expression of HIF-1α/VP16 augments the in-vitro and in-vivo arteriogenic effects of MSCs and may represent a novel approach for therapeutic arteriogenesis.

Condensed Abstract

MSCs from patients undergoing stem-cell therapy were either transduced with Ad.HIF-1α/VP16 vector, or exposed to normoxia or 1% O_2. VEGF and bFGF levels increased significantly in the conditioned media (MSC^{CM}) from HIF-1α/VP16-transduced MSCs compared to controls. When compared to MSC^{CM} from MSCs under normoxia or hypoxia, MSC^{CM} from transduced MSCs increased endothelial and smooth muscle cell proliferation and migration. In a model of hindlimb ischemia, adductor muscle injection of HIF-1α/VP16-transduced MSCs increased collateral perfusion, improved limb function, reduced calf muscle atrophy and increased adductor muscle levels of VEGF compared to controls.

Keywords: Marrow stromal cells, angiogenesis, gene therapy

The disappointing results of randomized clinical trials of single angiogenic agents - such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)[1-3] - have led investigators to pursue alternative strategies to enhance perfusion to ischemic tissues. One strategy is the utilization of angiogenic cells, such as multipotent progenitor cells, endothelial progenitor cells, and marrow stromal cells.[4-10] Although cell therapy may overcome some of the limitations of single angiogenic agent therapy, there are data to suggest that cell therapy itself may also have inherent limitations, including low rates of transplanted cell survival, and impairment of angiogenic potential in cells derived from senescent subjects.[11]

One approach to this problem is to genetically engineer such cells to enhance their angiogenic potential. For example, transduction of endothelial progenitor cells with $VEGF_{164}$ reduced the number of cells required to improve ischemic limb salvage when injected into athymic nude mice.[12] MSCs--recently demonstrated to enhance collateral remodeling in response to hindlimb ischemia--exert their effects mainly through paracrine mechanisms with release of a wide array of cytokines.[13] Central to regulating the expression of many of these angiogenic cytokines is the hypoxia inducible factor (HIF) family of transcription factors.[14] Given the critical angiogenic regulatory role of HIF, we hypothesized that *overexpression of a constitutively active form of a hypoxia inducible factor-1α analogue - HIF-1α/VP16 - enhances the arteriogenic potential of marrow stromal cells.* The purpose of the current study is to examine the validity of this hypothesis.

Methods

Cell Preparation

Normal human MSCs (derived from a single 19-year old healthy donor) were purchased from Clonetics (Walkersville, MD) and cultured in Dulbecco's Modified Eagles Medium (DMEM) with 20% fetal calf serum and 1% penicillin-streptomycin (DM-20). These cells were previously demonstrated to express surface markers typical of MSCs.[15] Passages 3-5 were used for in-vitro experiments. In addition, MSCs from 8 patients undergoing a phase 1 trial of autologous bone-marrow therapy for ischemic heart disease at our institution were cultured as previously described.[16] Cells were cultured in 20% O_2 and 5% CO_2 during normoxia experiments, and in 1% O_2 and 5% CO_2 for hypoxia experiments.

Human umbilical vein endothelial cells (ECs, ATCC, Manassas, VA) were cultured in endothelial growth media-2 (EGM-2; Clonetics). Human aortic smooth muscle cells (SMCs, ATCC) were cultured in Medium-199 supplemented with 10% serum and 1% penicillin-streptomycin (M-10). Passages 3-8 were used for in-vitro experiments.

Murine MSCs and ECs were prepared as previously described.[9] For injection, cells were recovered and resuspended in DM-20 at a concentration of $3x10^5$ cells per 50μl.

Cell transduction

Multiplicity of infection (MOI) curves were constructed for hMSCs and mMSCs using replication-deficient recombinant adenoviruses carrying the gene encoding green

fluorescent protein (Ad.GFP) under the control of the cytomegalovirus immediate-early promoter (CMV). MSCs were plated at a density of 10,000 cells per well (subconfluent). Incremental concentrations of virus over the range of 2 to 2000 multiplicity of infection (MOI) were applied for 2-hours in 0.2% DM, followed by washing and resuspension in DM-20. After 48-hours, cells were examined using a Nikon inverted fluorescence microscope and GFP-positive cells counted as a percentage of total cells in 10 random fields.

To track viral protein expression in-vivo, MSCs were incubated with Ad.GFP at an MOI of 100 for 2-hours, and immediately injected into the adductor muscle (24-hours post-surgery). To follow the fate of injected GFP+/MSCs, sections of adductor and calf muscle harvested at day-14 were examined using a Nikon inverted fluorescence microscope. The Ad.HIF-1α/VP16 was generously supplied by Genzyme Corporation (Framingham, MA) and also utilizes a CMV promoter to drive HIF-1α expression. Cells were transduced with Ad.HIF-1α/VP16 at an MOI of 150 and injected intra-muscularly 24-hours later.

RNA Preparation

Total RNA was extracted from Ad.HIF-1α/VP16 transduced hMSCs, and normoxia and hypoxia exposed (72-hours) hMSCs (2 plates per analysis) using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was cleaned using a RNeasy mini kit (Quiagen, Valencia, CA) and stored at –80°C. Two micrograms of total RNA underwent reverse transcription using Promega reverse transcription reagents and a vector specific reverse

primer. All samples were analyzed on an ABI 7700 instrument in a real-time semi-quantitative "TaqMan" PCR reaction specific for HIF-1α/VP16 vector. Copy number values were determined by comparing sample results to a standard curve made from log serial dilutions of the plasmid pHIF-1α/VP16 (101 to 105 copies).

Human Conditioned Media Collection, Preparation and Analysis

For ELISA, conditioned media was collected from hMSCs cultured under normoxia (MSC^{CM}), hypoxia (hypoxic-MSC^{CM}) or from cells transduced with Ad.HIF1α/VP16 (transduced-MSC^{CM}). After 24-hours of culture, media was centrifuged and passed through a 0.3μm filter. The concentration of vascular endothelial growth factor (VEGF) basic fibroblast growth factor (BFGF), placental growth factor (PlGF) and monocyte chemoattractant protein-1 (MCP-1) in the conditioned media was measured using sandwich ELISA kits (R&D systems, Minneapolis, MN). ELISA values were corrected for total cell protein (MicroBCA protein assay, Pierce, Rockford, IL). For cell proliferation and migration assays, conditioned media was collected for 72-hours and filtered as above.

Cell Proliferation and Migration Assays

ECs or SMCs (1×10^4/well) were plated in 24-well plates in DMEM with 0.1% serum for 24-hours to arrest mitosis. To study cell proliferation, the 0.1% DMEM was replaced with DM-20, MSC^{CM}, hypoxic-MSC^{CM} or transduced-MSC^{CM} and cultures continued for 72-hours. Cells were recovered and counted using a Coulter counter. Data are reported as the mean % change in proliferation when compared with control media (DM-20).

EC and SMC migration assays were performed using Transwell culture chambers (Costar, Corning, NY). Cells were suspended in DMEM supplemented with 2% serum and placed in the top chamber ($1x10^5$/well). DM-20, MSC^{CM}, hypoxic-MSC^{CM} or transduced-MSC^{CM} was added to the lower chamber and cells were incubated overnight. The top layer of the membrane was then scraped gently to remove any cells, and cells on the lower surface of the membrane stained using Hema-3 staining kit (Biochemical Sciences, Swedesboro, NJ). Ten random fields per membrane were counted. Data is reported as the mean % or fold change in proliferation when compared with control media (DM-20).

Animal Surgery and MSC Injection

All animal interventions were approved by the Animal Care and Use Committee of the MedStar Research Institute. Under narcosis, 12-week old Balb/C mice underwent right femoral artery ligation just proximal to the popliteal bifurcation. Twenty-four hours later mice were divided into 4 groups and received adductor muscle injections of either murine endothelial cells (EC, $1x10^5$ cells, n=8), murine MSCs (MSC, $1x10^5$ cells in 50µl DM-20, n=8), Ad.HIF-1α/VP16-transduced mMSCs (trMSC, $1x10^5$ cells, n=8) or Ad.HIF-1α/VP16 alone (Ad.HIF, $1x10^{10}$ viral particles, n=10). Injections were performed in 6 sites in the right adductor muscle adjacent to, and within 1mm proximally or distally of the ligation site. The dose of MSCs was carefully chosen based on previous experiments demonstrating a clear MSC dose-response relationship. Injection of $1x10^6$ MSCS resulted in a significant increase in hindlimb flow which genetic alteration was unlikely to

enhance. However, injection if a 10x lower dose ($1x10^5$) MSCs, resulted in minimal flow increase, allowing the potential for this effect to be augemtned by genetic engineering.

Animal Follow-up Analysis (Circ Res)

Laser Doppler Perfusion Imaging (LDPI) (Moor Instruments, Wilmington, DE) recorded blood flow recovery. For consistent measurements, imaging was performed after limb hair was removed, and after mice had been placed on a heating plate at 37°C to minimize temperature variation. Calculated perfusion is expressed as a ratio of the ischemic to normal limb. Previous data suggest a close linear relationship between recovery of perfusion as assessed by LDPI, and positive remodeling of adductor collateral vessels.[17]

Functional assessment of the ischemic limb was performed using a modification of a clinical standard score.[18] A semi-quantitative assessment of ambulatory impairment of the ischemic limb was performed serially (0 = flexing the toes to resist gentle traction on the tail, 1 = plantar flexion, 2 = no dragging but no plantar flexion, 3 = dragging of foot). A blinded observer assigned all scores.

Immunoblotting

Muscle samples were harvested at 7-days post-injection and protein extracted. Forty micrograms of proteins were separated using SDS-PAGE gels (10%) and blotted onto nitrocellulose (Invitrogen, Carlsbad, CA). After blocking with 10% milk, membranes were incubated with primary antibodies to VEGF (1:1000, Chemicon, Temecula, CA) or

α-tubulin (1:2000, Santa Cruz, Santa Cruz, CA). Membranes were developed with an enhanced chemiluminescence kit (Pierce, Rockford, IL).

Data and Statistical Analysis

Expression analysis data was verified by performing experiments in duplicate. All ELISA, immunoblotting, and cell studies were performed at least in triplicate. All results are presented as means ± SEM. Statistical significance was evaluated using an unpaired Student t-test, or ANOVA where indicated. A p-value of less than 0.05 was considered significant.

Results

Marrow Stromal Cell Transduction

Both human and murine MSCs were efficiently transduced with Ad.GFP at relatively low MOIs (Figure 1a-1c). At an MOI of 100, over 95% of cells were successfully transduced, although cytopathic effects were observed with MOIs above 500. RT-PCR confirmed successful introduction of the HIF-1α/VP16 construct into hMSCs, with 4,765 copies of the transgene per microgram of total RNA (Figure 1d).

Cytokine Release from MSCs

Under hypoxic stress, normal human donor MSCs released significantly more VEGF compared to normoxia (415pg/mg protein in normoxia vs. 724pg/mg in hypoxia, $p<0.05$). However, in comparison, transduction with Ad.HIF-1α/VP16 increased VEGF release fourfold (2,540pg/mg, $p<0.01$ vs. both controls, Figure 2a). Increasing Ad.HIF-1α/VP16

MOI also significantly increased VEGF release ($p<0.01$ for trend, Figure 2b). Similar results were obtained with MSCs derived from patients with ischemic heart disease. Mean VEGF levels increased from 630pg/mg under normoxia to 1150pg/mg under hypoxic stress ($p<0.05$), and to 3,023pg/mg following transduction with Ad.HIF-1α/VP16 ($p<0.01$ vs. both controls, Figure 2c). All patients' cells appeared to be successfully transduced regardless of age or presence of cardiovascular risk factors. Of note, one of the patients (triangle; Figure 2c), who expressed low VEGF levels under normoxic and hypoxic conditions, expressed VEGF levels that were considerably above the mean after Ad.HIF1α/VP16 transduction.

The release of bFGF was also augmented by Ad.HIF-1α/VP16 transduction (6,959pg/mg vs. 2,240pg/mg in normoxia vs. 4,120pg/mg in hypoxia, $p<0.05$ vs. normoxia, Figure 2d). Importantly, Ad.HIF-1α/VP16 transduction did not impair MSC release of other important arteriogenic cytokines (not under the control of HIF signaling) including PlGF and MCP-1 (Figure 2e and 2f).

Effects of Conditioned Media on Endothelial and Smooth Muscle Cell Proliferation and Migration

To examine whether MSC transduction with Ad.HIF-1α/VP16 exhibited biologic effects relevant to collateral remodeling, a series of EC and SMC proliferation assays were performed. Transduced-MSC^{CM} significantly enhanced EC proliferation over MSC^{CM} and hypoxic-MSC^{CM} (290% vs. 31% vs. 79% respectively, $p<0.01$ vs. both controls, Figure 3a). Similarly, although to a lesser degree, transduced-MSC^{CM} significantly enhanced

SMC proliferation over control (82% vs. 26% vs. 58% respectively, $p<0.05$ vs. MSC^{CM}, Figure 3b).

To further examine the biological effects of MSC transduction, EC and SMC migration assays were performed. Transduced-MSC^{CM} induced a 2-fold increase in EC migration compared to MSC^{CM} (1132% vs. 529%, $p<0.01$, Figure 3c). Transduced-MSC^{CM} also exerted an increased chemoattractant effect on SMCs compared to control, although generally the effects of conditioned media on SMC migration were weak (32% vs. 84%, $p<0.05$, Figure 3c).

In-Vivo Analysis of Ad.HIF-1α/VP16-Transduced MSC Injection

Having established that transduction of MSCs increased release of several important arteriogenic cytokines, and that this maneuver led to increases in in-vitro biologic effects relevant to collateral flow, we proceeded to investigate whether Ad.HIF-1α/VP16-transduced MSCs exerted a beneficial effect on in-vivo collateral development. In mice receiving ECs, flow returned to ~40% of the non-ischemic limb by day 14. Although there were trends for an increase in flow recovery in mice receiving Ad.HIF-1α/VP16 and MSCs, these failed to reach statistical significance (51% and 56% respectively, Figure 4b). In contrast, in those mice receiving transduced MSCs there was a significant improvement in flow by day-3, which was maintained for the duration of the study ($p<0.05$ by ANOVA). When compared to a previous study of MSC injection to enhance collateral flow, injection of $1x10^5$MSCs transduced with Ad.HIF-1α/VP16 resulted in similar flow recovery to a dose of $1x10^6$ non-transduced MSCs.[9] Representative flow

images are displayed in figure 4c. GFP+ cells are displayed in Figure 4d confirming in-vivo MSC expression of viral products.

The improved flow recovery observed in mice receiving Ad.HIF-1α/VP16-transduced MSCs was associated with improved hindlimb function (figure 5a). Improved flow recovery also attenuated the calf muscle atrophy observed following femoral artery ligation. In control mice, muscle loss was significantly greater than in those mice receiving Ad.HIF-1α/VP16-transduced MSCs (figure 5b). Immunoblotting confirmed that injection of transduced MSCs led to local increases in adductor muscle VEGF protein levels compared to mice receiving MSCs, Ad.HIF-1α/VP16, or ECs (Figure 5c).

Discussion

The enthusiasm for single angiogenic agent therapy--generated by multiple positive animal studies and the subsequent results of small non-randomized phase-I clinical trials--has recently waned following the disappointing results of randomized clinical trials.[1-3] One possible reason for these negative results is the need for multiple cytokines to enhance collateral development. Animal models demonstrate that the tissue response to arterial luminal narrowing is a complex process with multiple cytokines involved, acting in a coordinated time and concentration-dependent manner.[19-21] These cytokines not only have individual effects, but one cytokine may potentiate (or inhibit) the effect of another.[22-25] In light of these data, delivery of a single angiogenic protein may be inadequate to optimally improve collateral development. In addition, several

cardiovascular risk factors may negatively impact on tissue responses to ischemia and as such may also potentially inhibit responses to angiogenic therapy.[26-28]

As a result of these data, investigators have pursued alternative strategies, such as the utilization of cell therapy to enhance collateral flow. Previous studies suggest than cell therapy may influence collateral remodeling though multiple pathways, and in particular through the secretion of multiple arteriogenic cytokines. However, cell therapy itself may also have inherent limitations. Cardiovascular risk factors such as aging impair the effectiveness of cells derived from the older patient in whom autologous cells are to be injected[11,29] Transplanted cells may also have low survival rates, significantly impacting on their beneficial effects. Furthermore, even if BM cell therapy is more efficacious than single agent therapy, it may still be insufficient to overcome the inhibitory effects of multiple cardiovascular risk factors on collateral development.

Genetic engineering of cells for therapy may be one option to address such limitations. One approach is to introduce genes that enhance transplanted cell survival. Overexpression of the pro-survival factor Akt in MSCs injected into myocardial scar increased myocardial tissue regrowth compared to MSCs transduced with a reporter gene.[30] An alternative approach is to introduce genes that augment the arteriogenic potential of cells. Transduction of skeletal myoblasts with $VEGF_{165}$ increased capillary density in the vicinity of the transplanted cells compared to non-transduced cells.[31] Similarly, transduction of endothelial progenitor cells with $VEGF_{164}$ reduced by thirty-

fold the number of cells required to improve ischemic limb salvage when compared to previously performed studies.[12]

In this investigation we determined whether cellular overexpression of HIF-1α augments the arteriogenic potential of MSCs. Because of the lability of HIF-1α protein in the absence of hypoxia, a form of the protein resistant to degradation was developed (Genzyme Corporation). In this construct the VP16 domain (derived from the herpes simplex virus) replaces the ubiquination site of the expressed gene product, thus abolishing the proteasomal-mediated degradation of the protein.[32] The transcriptional activity of HIF-1 derives from its capacity to bind to the promoter of many genes involved in the response of the cell to hypoxia, including all isoforms of VEGF, insulin-like growth factors, angiopoietins-1 and 4, and nitric oxide synthases.[14,33-34] Thus, HIF-1α gene therapy has the theoretical potential to be more efficacious in augmenting tissue perfusion than gene therapy with single angiogenic agents such as $VEGF_{165}$.

The present study does not address the question of efficacy of gene therapy vs. single angiogenic agent therapy. However, it does demonstrate that introduction of the HIF-1α/VP16 construct into MSCs enhances their therapeutic potency. Importantly, the study also demonstrates that the introduction of HIF-1α/VP16-transduced MSCs is significantly more effective in restoring limb perfusion than direct intramuscular injection of Ad.HIF-1α/VP16.

There are several potential explanations for this latter finding. Firstly, introduction of the HIF-1α/VP16 construct into different cells may lead to variable responses. In particular, introduction of HIF-1α/VP16 into cells--such as MSCs--whose major effects are exerted through paracrine signaling, may be particularly efficacious in restoring perfusion. A second explanation relates to doubts regarding the effectiveness with which adenoviruses can transduce skeletal muscle cells. Cellular adenoviral entry is facilitated via the Coxsackie-Adenovirus-Receptor complex (CAR), and although cardiomyocytes express CAR in high concentration, skeletal muscle cells express very low concentrations.[35] Indeed, this phenomenon was postulated as one explanation for the negative result of the RAVE trial.[36] As such, intramuscular delivery of genetically engineered cells may be a more effective way of delivering gene products to peripheral muscle than can be achieved by direct injection of adenoviral vectors. In the current study, many MSCs transduced with the gene encoding GFP were observed to express their transgene for at least 21-days following injection, clearly demonstrating this concept in-vivo.

In summary, transduction of human MSCs with the HIF-1α/VP16 construct increases in-vitro VEGF and bFGF secretion, and increases the paracrine effects of MSCs on endothelial and smooth muscle cell proliferation and migration. In a murine model of hindlimb ischemia, adductor muscle injection of HIF-1α/VP16-transduced MSCs increases collateral perfusion, improves limb function, and reduces calf muscle atrophy compared to control. Thus, although the optimal strategy for enhancing collateral development is yet to be identified, these results suggest that cell therapy, when combined with genetic manipulation of the cells, may hold great promise.

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Figure Legends

Figure 1. a) MOI curves for murine MSCs indicating successful transduction of almost all cells above an MOI of 50. b) Similar MOI curve for human MSCs. c) Representative fluorescent image of GFP+ human MSCs. d) RT-PCR data confirming MSC transduction with the HIF-1α/VP16 construct.

Figure 2. a) In-vitro release of VEGF, from hMSCs (MSC^{CM}, $hyMSC^{CM}$, $trMSC^{CM}$ represent conditioned media derived from MSCs under normoxia, hypoxia and following HIF-1α/VP16 transduction respectively; *$p<0.05$ vs. MSC^{CM}, †$p<0.01$ vs. both controls). b) Dose-response curve for Ad.HIF-1α/VP16 transduction of MSCs (†$p<0.01$for trend). c) Successful transduction of hMSCs derived from patients undergoing bone-marrow cell therapy for ischemic heart disease. Open shapes represent individual patient data, with the closed circles representing mean data for all patients. d) In-vitro release of bFGF from hMSCs (*$p<0.05$ vs. MSC^{CM}). e) In-vitro release of PlGF from hMSCs. f) In-vitro release of MCP-1 from hMSCs.

Figure 3. a) EC proliferation in response to MSC^{CM}, $hyMSC^{CM}$, $trMSC^{CM}$. Data are expressed as percent increase over DM-10 (†$p<0.01$ vs. MSC^{CM} and $hyMSC^{CM}$). b) SMC proliferation in response to MSC^{CM}, $hyMSC^{CM}$, $trMSC^{CM}$; (*$p<0.05$ vs. MSC^{CM}). c) EC and SMC migration in response to MSC^{C} and $trMSC^{CM}$. Data are expressed as percent increase over DM-10 (†$p<0.01$ vs. MSC^{CM}, and *$p<0.05$ vs. MSC^{CM}).

Figure 4. a) Photograph indicating the suture around the femoral artery (white arrow) in the distal adductor region and the region of MSC injection (black arrows). b) LDPI expressed as a percentage of the normal limb. Flow recovery in animals receiving Ad.HIF-1α/VP16-transduced MSCs (trMSC) was significantly better than that seen in control animals (EC - endothelial cell control, MSC - non-transduced MSCs, Ad.HIF - Ad.HIF-1α/VP16; *$p<0.05$ vs. all controls by ANOVA). c) Representative LDPI images of flow recovery in a mouse receiving trMSCs versus a mouse receiving MSCs. Red is highest velocity, green intermediate, and blue, lowest velocity. d) x10 fluorescent image displaying GFP+ MSCs 14-days after injection confirming cell viability, and ability to express viral gene products in-vivo.

Figure 5. a) Effects of treatment group on ambulatory function (*$p<0.05$ vs. EC, †$p<0.01$ vs. EC and Ad.HIF, ‡$p<0.05$ vs. MSC). b) Effects of treatment group on calf atrophy (*$p<0.05$ vs. EC, †$p<0.01$ vs. EC and Ad.HIF, ‡$p<0.05$ vs. MSC). c) Western blotting (day-7) demonstrating that Ad.HIF-1α/VP16-transduced MSC injection increases local production of VEGF compared to control groups.